PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

CHIMERICAL PEPTIDE-NUCLEIC ACID FRAGMENT, PROCESS FOR PRODUCING THE SAME AND ITS USE FOR APPROPRIATELY INTRODUCING NUCLEIC ACIDS INTO CELL ORGANELLES AND CELLS

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Inventors: Peter Seibel
Andrea Seibel

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Albert P. Halluin, Esq.
PENNIE & EDMONDS
1155 Avenue of the Americas
New York, New York 10036-2711

(415) 854-3660

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CHIMERICAL PEPTIDE-NUCLEIC ACID FRAGMENT, PROCESS FOR PRODUCING THE SAME AND ITS USE FOR APPROPRIATELY INTRODUCING NUCLEIC ACIDS INTO CELL ORGANELLES AND CELLS

This is a national phase filing of the Application No. PCT/DE95/00775, which was filed with the Patent Corporation Treaty on June 11, 1995, and is entitled to priority of the German Patent Application P 44 21 079.5, filed June 16, 1994.

I. FIELD OF THE INVENTION

This invention relates to a chimerical peptide-nucleic acid fragment, the process for producing the same and its use for appropriately introducing nucleic acids into cell organelles and cells.

II. BACKGROUND OF THE INVENTION

It is now that cellular membrane systems are largely impermeable to nucleic acids. However, cell membranes can be overcome very efficiently by physical processes (transformation) and biological processes (infection). Transformation, i.e., the direction absorption of the naked nucleic acid by the cells, is preceded by cell treatment. There are various methods available for the production of these 'competent cells'. Most processes are based on the observations made by Mandel and Higa (M. Mandel *et al.*, (1970), "Calcium-dependent bacteriophage DNA infection", *J. Mol. Biol.* 53: 159-162), who could show for the first time that yields resulting from the absorption of lambda-DNA by bacteria can be increased fundamentally in the presence of calcium chloride. This method is also used successfully for the first time by Cohen *et al.* (S.N. Cohen *et al.* (1972), "Nonchromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor DNA", *Proc. Natl. Acad. Sci. U.S.A.* 69:2110-2114) for plasmid DNA and was improved by many modifications (M. Dager *et al.* (1979), "Prolonged incubation in calcium chloride improves the competence of Escherichia coli cells", *Gene* 6:23-28). Another transformation method is based on the observation that high-frequency alternating fields may break up cell membranes

(electroporation). This technique can be used to introduce naked DNA into not only prokaryotic cells but also eukaryotic cell systems (K. Shigekawa *et al.* (1988), "Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells", *Biotechniques* 6:742-751). Two very gentle methods of introducing DNA into eukaryotic cells were developed by Capecchi (M.R. Capecchi (1988)), "High efficiency transformation by direct microinjection of DNA into cultured mammalian cells" *Cell* 22:479-488) and Klein *et al.* (T.M. Klein *et al.* (1987), "High velocity microprojectiles for delivering nucleic acids into living cells", *Nature* 327:70-73): They are based on the direct injection of the DNA into the individual cell (microinjection), on the one hand, and on the bombardment of a cell population with microprojectiles consisting of tungsten, to the surface of which the corresponding nucleic acid was bound ('shotgun'). The biological infection methods proved their value parallel to the physical transformation of cells. They include particularly the high efficient viral introduction of nucleic acids into cells (K.L. Berkner (1988), "Development of adenovirus vectors for the expression of heterologous genes", *Biotechniques* 6:616-629; L.K. Miller (1989), "Insect baculoviruses: powerful gene expression vectors", *Bioessays* 11:91-95; B. Moss *et al.* (199), "Product review. New mammalian expression vectors", *Nature* 348:91-92) and the liposome mediated lipofection (R.J. Mannino *et al.* (1988), "Liposome mediated gene transfer", *Biotechniques* 6:682-690; P.L. Felgner *et al.* (1987), "Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure", *Proc. Natl. Acad. Sci. U.S.A.* 84:7413-7417). All methods described so far deal with the overcoming of the prokaryotic or eukaryotic plasma membrane by naked or packaged nucleic acids. While the site of action is reached already when the nucleic acid are introduced into the prokaryotic cell, further biochemical processes take place in a compartmentalized eukaryotic cell, which support the penetration of the nucleic acid into the nucleus under certain conditions (e.g. viral route of infection in the case of HIV). Analogous infective processes in which exogenous nucleic acids are actively introduced into other cell organelles (e.g., into mitochondria) have not been described so far.

In addition to the introduction of the nucleic acid into the cell and cell organelle, respectively, the transcription and above all the replication of the introduced nucleic acid

play a decisive part. In this connection, it is known that the DNA molecules may have a special property which permits duplication in a cell under certain conditions. A special structural element, the origin of the DNA replication (ori, origin), adds thereto. Its presence provides the ability of DNA replication (K.J. Marians (1992), "Prokaryotic DNA replication", *Annu. Rev. Biochem.* 61:673-719; M.L. DePamphilis (1993), "Eurkaryotic DNA replication: anatomy of an origin", *Annu. Rev. Biochem.* 62:29-63; H. Echols and M.F. Goodman (1991), "Fidelity mechanisms in DNA replication", *Annu. Rev. Biochem.* 60:477-511). The strictly controlled process of DNA replication starts in E. coli e.g., when a protein is bound (K. Geider and H. Hoffman Berling (1981), "Proteins controlling the helical structure of DNA", *Annu. Rev. Biochem.* 50:233-260) to the highly specific initiation site thus defining the starting point of a specific initiation site thus defining the starting point of a specific RNA polymerase (primase). It synthesizes a short RNA strand (~10 nucleotides, 'primer') which is complementary to one of the DNA template strands. The 3' hydroxyl group of the terminal ribonucleotide of this RNA chain serves as a 'primer" for the synthesis of new DNA by a DNA polymerase. DNA-untwisting proteins unwind the DNA double helix (J.C. Wang (1985), "DNA topoisomerases", Annu. Rev. Biochem. 54:665-697). The separated individual strands are stabilized by DNA-binding proteins as regards their conformation (J.W. Chase and K.R. Williams (1986), "Single-stranded DNA binding proteins required for DNA replication", *Annu. Rev. Biochem.* 55:103-136) to enable proper functioning of the DNA polymerases (T.S. Wang (1991), "Eukaryotic DNA polymerases", *Annu. Rev. Biochem.* 60:513-552). A multienzyme complex, the holoenzyme of DNA-polymerase-III, synthesizes the majority of the new DNA. The RNA portion of the chimerical RNA-DNA molecule is then split off the DNA polymerase III. The removal of the RNA from the newly formed DNA chains creates gaps between the DNA fragments These gaps are filled by the DNA-polymerase I which can newly synthesize DNA from a single-stranded template. While one of the two newly synthesized DNA strands is synthesized continuously (5'-3' direction, leader strand), Ogawa and Okazaki observed that a majority of the newly synthesized opposite strand (3'-5' direction, delayed strand) was synthesized from short DNA fragments (T. Ogawa and T. Okazaki (1980), "Discontinuous DNA replication", *Annu. Rev. Biochem.*

49:421-457). Here, what is called primases initiate the onset of the DNA synthesis of the opposite strand by the synthesis of several RNA primers. When the replication proceeds, these fragments are freed from their RNA primers, the gaps are closed and covalently linked with one another to give extended daughter strands by the DNA ligase. Two chromosomes form after the termination of the replication cycle.

As opposed thereto, the DNA replication is controlled by many plasmids via a replication origin which dispenses with the synthesis of the delayed strand (3'-5' direction) and can initiate the synthesis of two continuous DNA strands bidirectionally (each in the 5'-3' direction along the two templates). The precondition for a complete DNA replication is here the cyclic form of the nucleic acid. It ensure that at the end of the new synthesis of the complementary DNA strands the DNA polymerases return to the starting point again where now ligases guarantee the covalent linkage of the ends of the two newly synthesized daughter strands.

Smallpox viruses represent an interesting form of linear-cyclic nucleic acids: because of what is called 'hairpin loops' at the ends of their linear genomes they have a cyclic molecule structure while maintaining a predominantly linear conformation (D.N. Black *et al.* (1986), "Genomic relationship between capripoxviruses", *Virus Res.* 5:277-292; J.J. Esposito and J.C. Knight (1985) "Orthopoxyvirus DNA: a comparison of restriction profiles and maps", *Virology* 143:230-251). Covalently closed "hairpin" nucleic acids were not only found in smallpox viruses but also described for the ribosomal RNA from Tetrahymena (E.H. Blackburn and J.T. Gall (1978), "A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena", *J. Mol. Biol.* 120:33-53) and the genomes of the parvoviruses (S.E. Straus *et al.* (1976), "Concatemers of alternating plus and minus strands are intermediates in adenovirus-associated virus DNA synthesis", *Proc. Natl. Acad. Sci. U.S.A.* 73:742-746; P. Tattersall and D.C. Ward (1976), "Rolling hairpin model for the replication of parvovirus and linear chromosomal DNA", *Nature* 263:106-109).

However, my means of the formerly known plasmids or nucleic acid constructs it is not possible to appropriately introduce nucleic acids into cells or cell organelles via the protein import route. But this is e.g. a precondition for treating genetically changes for the mitochondrial genomes of patients suffering from neuromuscular and

neurodegenerative diseases or carrying out an appropriate mutagenesis in mitochondria or other cell organelles.

III. SUMMARY OF THE INVENTION
[TRANSLATION WILL BE PROVIDED]

IV. BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is explained particularly the figures, wherein:

Figure 1 depicts a signal peptide of the ornithine transcarbamylase of rats as well as a DNA sequence suitable for the introduction. Top: signal peptide of the ornithine transcarbamylase of rates (32 amino acids), extended by 10 N-terminal amino acids of the matured protein and an additional cysteine as linkage site. The peptide sequence is shown in the international one-letter code; middle: a partially palindromic DNA sequence suitable for the introduction and consisting of 39 nucleotides having an amino-modified T at nucleotide position 22; bottom: marked secondary structure of the oligonucleotide having an overhanging 5' end and a modified nucleotide in the vertex of the 'loop'.

Figure 2 depicts the structure of the amino-modified 2-deoxythymidine, R: nucleic acid residues.

Figure 3 depicts a diagram of chimerical peptide-nucleic acid fragment, consisting of amino-modified oligonucleotide (39 nucleotides) with marked 'hairpin loop', cross-linker and signal peptide. CL: Cross=linker.

Figure 4 the electrophoretic separation of the linkage product resulting from amino-modified oligonucleotide (39 nucleotides), m-maleimidobenzoyl-N-hydroxy-succinimide ester (MBS) and signal peptide of the ornithine transcarbamylase of rates (42 amino acids, extended by a cysteine at the C terminus).

Figure 5A depicts a flow diagram of the peptide-DNA fusion, cloning, amplification and linkage of the transcribable and processable mitochondrial tRNA gene to be introduced (S. Anderson et al. (1981), "Sequence and organization of the human mitochondrial genome", *Nature* 290:457-465). CL: cross-linker (MBS); MCS: multiple cloning site of pBluescript^{R} (Stratagene), mtTF: binding site of the

mitochondrial transcription factor; RNA-Pol: binding site of the mitochondrial RNA polymerase; tRNA Leucin: gene of the mitochondrial transfer RNA for leucine (UUR); *Sac II, Apa I, Eco RI*: sites for restriction endonucleases; the cloned mitochondrial sequences were numbered in accordance with the publishes sequence of the human mitochondrial genome (S. Anderson *et al.* (1981), "Sequence and organization of the human mitochondrial genome", *Nature* 290:457-465)

Figure 5B depicts the sequence of the cloned $tRNA^{Leu(UUR)}$ gene.

Figure 6A and 6B depict a presentation of the ^{32}p radiation of the DNA as well as the enzyme activities for adenylate kinase, cytochrome c oxidase and malate dehydrogenase (y axes) in 11 fractions (x axes) of a mitochondria-sucrose gradient density centrifugation. The portion of the particular radiation/enzyme activity, expressed as percentage of the total radiation/enzyme activity which was plotted against the gradient is illustrated. ADK: adenylate kinase; COX: cytochrome c oxidase; MDH: malate dehydrogenase.

Figure 7A and 7B depicts a presentation of the ^{32}p radiation of the DNA as well as the enzyme activities for adenylate kinase, cytochrome c oxidase and malate dehydrogenase (y axes) in 11 fractions (x axes) of a mitoplast-sucrose gradient density centrifugation. The portion of the particular radiation/enzyme activity, expressed as percentage of the total radiation/enzyme activity which was plotted against the gradient, is illustrated. ADK: adenylate kinase; COX: cytochrome c oxidase; MDH: malate dehydrogenase.

Figure 8 depicts the cloning of the nucleic acid portion of the peptide-nucleic acid plasmid into pBluescript (plasmid 1). Using the two oligonucleotides (primers 1 and 2), the gene section of nucleotide 15903 to nucleotide 677 was amplified enzymatically from mitochondrial HeLa DNA (comprises: promoter characterized by the binding sites for the mitochondrial transcription factors and the RNA polymerase; replication origin characterized by what is called 'conserved sequence blocks'; regulation of the DNA replication characterized by the 'TAS' motifs). Since the oligonucleotides contain recognition sequences for the restriction endonucleases *Xho* I and *Pst* I, the ends of the amplified nucleic acid can be modified such that they are compatible with a vector arm of pBluescript, on the one hand, and compatible with the

hybrid of the oligonucleotides MCS/TTS 1 and 2, on the other hand. In addition to a multiple cloning site, they also comprise a transcription termination sequence which is responsible for the regulated transcription. The ligation product is then transformed into *E. coli* XL 1. Following the plasmid isolation of insert-carrying *E. coli* colonies, the nucleic acids were subjected to RFLP and sequence analysis.

Figure 9 depicts the sequence of the oligonucleotides MCS/TTS 1 and 2. The oligonucleotides MCS 1 and 2 were prepared synthetically and comprise recognition sequences for nine different restriction endonucleases as well as a sequence motif which can suppress the transcription bidirectionally. The olignucleotides are complementary and can thus form a hybrid. The overhanging ends are part of the recognition sequences for the restriction endonucleases *Pst*I and *Bam* HI.

Figure 10 depicts the nucleotide sequence of the nucleic acid portion of the peptide-nucleic acid plasmid (plasmid 1).

Figure 11 depicts the cloning of the reporter gene into the nucleic acid portion of the peptide-nucleic acid plasmid into pBluescript (plasmid 2). Using the two oligonucleotides (primers 3 and 4), the gene section of nucleotide 1562 to nucleotide 3359 was amplified enzymatically from a DNA extract of a human CAP=resistant cell line (comprises: part of the 12 S rRNA gene, $tRNV^{Val}$ gene, 16 S $rRNA^{CAP+}$ gene, $tRNA^{Leu}$ gene, part of ND 1 gene). Since the oligonucleotides contain recognition sequences for the restriction endonucleases *Hind* III and *Bcl* I, the ends of the amplified nucleic acid can be modified such that they are compatible with the multiple cloning site (MCS) of the peptide-nucleic acid plasmid (plasmid 1). The ligation product is then transformed in *E. coli* XL 1 Blue. Following the plasmid isolation of insert-carrying *E. coli* colonies, the nucleic acids were subjected to the RFLP and sequence analysis and are available for the described experiments.

Figure 12 depicts the nucleotide sequence of the nucleic acid portion of the peptide-nucleic acid plasmid including the reporter gene (plasmid 2).

Figure 13A depicts the reaction run of the cyclization of the nucleic acid portion as well as the conjugation of the nucleic acid portion with a signal peptide. The nucleic acid portion of the peptide-nucleic acid plasmid can be obtained via a plasmid preparation or an enxymatic amplification. In both cases, the treatment with the

restriction endonuclease *Bsa* I results in an intermediate product capable of ligation. It can be reacted directly with the monomerized 'hairpin loops'. The reaction product is freed by an exonuclease III treatment from non-specific (non-cyclic) reaction products and educts, is purified and conjugated with the signal peptide via a cross-linker. As an alternative, one of the two 'hairpin loops' can first be conjugated with the signal peptide via a cross-linker before the cyclizing ligation reaction is carried out. A purification of the reaction product follows an exonuclease III treatment here as well.

Figure 13B depicts the structure and sequence of the 'hairpin loop' oligonucleotides HP 1 and 2.

Figure 14 depicts the monomerization of a 'hairpin loop' oligonucleotide. The synthetic 'hairpin loops' HP 1 and 2 can be monomerized by a thermal or alkaline denaturation. This figure shows a standard agarose gel: lane 1, molecular weight standard (ΦX 174 RF DNA treated with the restriction endonuclease *Hae* III), lane 2: HP 1, synthesis product; lane 3: HP 1, thermally monomerized.

Figure 15 depicts a ligation reaction between the nucleic acid portion of the peptide-nucleic acid plasmid (plasmid 2) and the 'hairpin loops' HP 1 and 2. This figure shows a standard agarose gel: lane 1, cloned nucleic acid portion of the peptide-nucleic acid portion in pBluescript treated with the restriction endonuclease *Bsa* I, lane 2: ligation of the reaction products resulting from lane 1 with the 'hairpin loops' HP 1 and 2; lane 3, treatment of the reaction production resulting from lane 2 with exonuclease III; lane 4, molecular weight standard (λDNA treated with the restriction endonucleases *HIND* III and *Eco* RI).

Figure 15B depicts the examination of the purified ligation product by a *Mae* III-RFLP analysis. This figure illustrates a standard agarose gel: lane 1, enzymatically amplified nucleic acid portion following a *Mae III* treatment; lane 2: purified ligation product of the enzymatically amplified nucleic acid portion following a *Mae* III treatment; lane 3: purified product of the plasmid DNA ligation following a *Mae* III treatment; lane 4, molecular weight standard (ΦX 174 RF DNA treated with the restriction endonuclease *Hae* III).

Figure 16 depicts the transcription and replication of the peptide-nucleic acid plasmid. This figure illustrates a standard agarose gel: lane 1, molecular weight

standard (λDNA treated with the restriction endonucleases *Hind* III and *Eco* RI); lane 2, untreated peptide-nucleic acid plasmid; lane 3: *in vitro*-obtained transcription products of the peptide-nucleic acid plasmid; lane 4: *in vitro*-obtained replication and transcription products of the peptide-nucleic acid plasmid; lane 5, *in vivo*-obtained replication and transcription products of the peptide nucleic acid plasmid; lane 6, untreated peptide-nucleic acid plasmid.

V. DETAILED DESCRIPTION OF THE INVENTION

It is the object of the present invention to develop a construct on a nucleic acid basis which permits the appropriate introduction of nucleic acids into cell and compartments of eukaryotic cells. Furthermore, a process is to be provided of how this construct can reach cell compartments or cells. In addition, the introduced nucleic acid should be such that it can also be incorporated as replicative nucleic acid via cellular protein import routes. Besides properties should be presented which result in a controlled transcription and/or replication in cells and in defined aimed compartments of cell, respectively. The process is to be used for the therapy of genetic diseases (changes of the mitochondrial genome) and for the appropriate mutagenesis in eukaryotic and prokaryotic cells. The invention is to meet the following demands:

- universal applicability
- cell-specific, compartment-specific and membrane-specific introduction behavior
- high degree of effectiveness
- low immunogenicity
- minimization of the infection risk
- the introduced nucleic acid (plasmid molecule) is to be replicatable
- the introduced nucleic acid (plasmid molecule) is to be transcribable
- the introduced nucleic acid (plasmid molecule) shall be resistant to nucleases
- the structure of the introduced nucleic acid (plasmid molecule) should be universally usable.

This problem is solved by the features of claims 1), 25), 54), 56), 58), 60) and 61). Advantageous embodiments follow from the subclaims.

In order to be able to appropriately carry a protein within a cell from the site of formation to another compartment or another cell organelle (e.g. the site of action), the protein is usually synthesized as a preprotein (R. Zimmermann et al. (1983), "Biosynthesis and assembly of nuclear-coded mitochondrial membrane proteins in Neurospora crassa", *Methods Enzymol.* 97:275-286). In addition to the matured amino acid sequence, the preprotein has what is called a signal sequence. This signal sequence is specific to the aimed compartment and enables that the preprotein can be recognized by surface receptors. The natural obstacle 'membrane" is then overcome by translocating the preprotein through the membrane by an active (several 'transport proteins' are involved in this process) or passive process (direct passage without involvement of further proteins). Thereafter, the signal sequence is usually separated on the site of action by a specific peptidase unless it is a constituent of the matured protein. The matured protein can now unfold its enzymatic activity.

The inventors have recognized that his mechanism can be utilized to appropriately transport nucleic acids across membranes. In this case, the nucleic acid is not subject to a restriction, i.e., it is possible to use every nucleic acid desired and known, respectively. For this purpose, a cell-specific, compartment-specific or membrane-specific signal sequence is linked with the desired nucleic acid, resulting in a chimerical peptide-nucleic acid fragment. In this context, it is known that the linkage between a nucleic acid and a peptide may occur via the α-amino group of a synthetic KDEL peptide, modified by ϵ-maleimidocapronic acid-N-hydroxysuccinimide ester (K. Arar *et al.* (1993), "Synthesis of oligonucleotide-peptide conjugates containing a KDEL signal sequence", *Tetrahedron Lett.* 34:8087-8090). However, this linkage strategy is completely unusable for the nucleic acid introduction into cell organelles and cells, since here the translocation should occur in analogy to the natural protein transport. Such a transport cannot be expected by clocking the α-amino group of a synthetic peptide by means of a nucleic acid. Therefore, the inventors chose linkage via a carboxy-terminal amino acid. On the one hand, this ensures a 'linear' linkage, on the other hand, the

free amino-terminal end of the signal peptide is thus available for the essential steps of the import reaction.

In order to be able to utilize the described transport mechanism also for the introduction of replicative and transcription-active nucleic acids, the nucleic acid is preferably integrated via a homologous recombination into an existing genome or is itself the carrier of the genetic elements, which ensures an autonomous initiation of replication and transcription. Only the latter variant complies with the criterion of universal applicability, since a recombination into an existing cellular genome is successful only under certain conditions and in select cells.

In this case, the use of cyclic DNA represents one possibility, since the DNA polymerases at the end of the new synthesis of the daughter strands return to the initial point thus guaranteeing a complete DNA replication. Although the use of a double-stranded cyclic plasmic meetings all physical criteria for a successful replication in every aimed compartment of the cell, this physical DNA form is confronted with the import pore size which is decisively involved in the appropriate translocation: Even the compact diameter of a superhelical plasmic can be compared with that of globular proteins, therefore, a translocation through a membrane system via the protein import route appears impossible. Here, an approach to a solution consists in the use of linear-cyclic DNA molecules having modified (cyclic) ends but only the diameter of linear DNA molecules. On the one hand, they are no obstacle for the import pore size; on the other hand, these linear-cyclic DNA molecules include all physical preconditions to be able to form replicative and transcription-active plasmids in the mitochondria.

The following is preferably required for the construction of the chimerical peptide-nucleic acid fragment according to the invention as well as for the construction of a replicative and transcription-active nucleic acid portion (plasmid):

- signal peptide and signal sequence, respectively, (cell-specific, compartment-specific, or membrane-specific)
- linkage agent
- nucleic acid (oligonucleotide) which may preferably comprise the following further information:

- information in the initiation and regulation of transcription and replication,
- information as to the termination of transcription and replication,
- multiple cloning site for a nucleic acid to be introduced (to be expressed) additionally,
- possible modifications, so that 'hairpin loops' can be added (cyclization of the ends) which permit linkage with the signal peptide.

The selection of the signal sequence depends on the membrane and membrane system, respectively, which is to be overcome and the aimed compartment of the cell (cell nucleus, mitochondrion, chloroplast) or the cell organelle which is to be obtained. Proteins which are to be introduced e.g. into one of the four mitochondrial compartments (outer mitochondrial membrane, intermembranous space, inner mitochondrial membrane, matrix space), have compartment-specific signal sequences. In general, signal sequences are chosen for the introduction of nucleic acids which contain a cell specific, compartment-specific or membrane-specific recognition signal thus directing the attached nucleic acid of its site of action (e.g., inner side of the inner mitochondrial membrane or matrix space). A selection can be made among signal sequences which can transport proteins in the presence or absence of a membrane potential. For the nucleic acid introduction, signal sequences which function irrespective of the membrane potential are preferred, e.g., the signal sequence of ornithine transcarbamylase (OTC) for the transport into the matrix space of the mitochondria (A.L. Horwich *et al.* (1983), "Molecular cloning of the cDNA coding for rate ornithine transcarbamylase", *Proc. Natl. Acad. Sci. U.S.A.* 80:4258-4262; J. P. Kraus *et al.* (1985), "A cDNA clone for the precursor of rate mitochondrial ornithine transcarbamylase: comparison of rate and human leader sequences and conservation of catalytic sites", *Nucleic. Acids. Res.* 13:943-952). Basically, the pure signal sequence suffices for the transport into the aimed compartment. However, preferable is to select signal sequences which additionally have a cell-specific or compartment-specific peptidase cleavage site. In the most favorable case, the "cleavage site' is within the

signal sequence but can also be attached thereto by additional amino acids to ensure the cleavage of the signal sequence when the aimed compartment has been reached (e.g., the signal sequence of human OTC can be prolonged by ten additional amino acids of the matured OTC). This ensures that the nucleic acid can be separated from the signal peptide in the aimed compartment, so that the action of the nucleic acid fully unfolds. The selected signal sequence is prepared biologically (purification of natural signal sequences or cloning and expression of the signal sequence in a eukaryotic or prokaryotic expression system) but preferably in a chemical-synthetic way.

In order to ensure a linear chemical linkage between nucleic acid and signal peptide, the signal peptide is linked via a linkage agent which is generally linked therewith via amino acids, preferably via amino acids having reactive side groups, preferably via an individual cysteine or lysine at the carboxy-terminal end of the signal peptide. A bifunctional cross-linker services as a linkage reagent, preferably a heterobifunctinal cross-linker which has a second reactive group, preferably an aminoreactive group, in addition to a thiol-reactive group at the signal peptide when a cysteine is used as the linkage site (e.g. m-maleinimidobenzoyl-N-hydroxy-succinimide ester, MBS and its derivatives).

The nucleic acid also has a linkage site which should be compatible with the selected cross-linker. When MBS is used, the oligonucleotide should have an amino function or thiol function. The linkage group of the nucleic acid can be introduced via the chemical synthesis of the ologonucleotide and is generally localized at the 5' end, at the 3' end, but preferably directly at a modified base, e.g., as 5' amino linker (TFA amino linker Amidite^R, 1,6-(n-trifluoroacetylamino)-hexyl-β-cyanoethyl-N,N-diisopropyl phosphoramidite, Pharmacia) or a 5' thiol linker (THIOL-C6 Phosphoramidit^R, MWG Biotech) at a free 5' hydroxy/phosphate group, as 3' amino linker (3' aminomodifier-C7-CPG-Synthesesäulen^R, MWG Biotech) at a free 3' hydroxy/phosphate group, but preferably as amino-modified base analog, preferably amino-modified deoxyrurindine (Amino-Modifier-dt^R, 5'-dimethoxy-trityl-5[N-(trifluoroacetylaminohexyl)-3-acrylimido]-2'-deoxyuridine, 3'-[2-cyanoethyl)-(N,N-diisopropyl)]phosphoramidite, Glen Research) within the sequence. In this case, the reactive group compatible with the cross-linker used is spaced from the 5' end or 3' end of the oligonucleotide or the modified base by

at least one C2-spacer unit, but preferably by a C6-spacer unit. The nucleic acid (oligonucleotide) including a reactive linkage group then comprises at least two nucleotides.

In order to increase the stability of the nucleic acid (oligonucleotide) over cellular and extracellular nucleases, the chemically synthesized nucleic acids can be protected by a sulfurizing reagent (Beaucage-Reagenz^R, MWG- Biotech). The phosphorus diester bonds of the nucleic acid are converted into phosphorus thioate bonds in the chemical synthesis. This oligonucleotide can then be used for the enzymatic amplification of nucleic acids, extended by further linkage reactions with other nucleic acids or used directly.

In order to directly use the chimerical peptide nucleic acid fragment, the nucleic acid (Oligonucleotide) should have a secondary structure that can be hybridized, preferably without internal homologies so as to be able to form a linear single-strand structure. This ensures that the nucleic acid (oligonucleotide) of the chimerical peptide-nucleic acid fragment can unfold a biochemical/therapeutic effect without further nucleic acid linkages.

However, for linkage with the signal sequence it is preferred to use nucleic acids (ogionucleotides) which have two further properties:

1. The sequence is preferably partially palindromic, has a blunt 5'-3' end ('blunt end'), an overhanging 3' end ('sticky end'), but has especially an overhanging, phosphorylated 5' end ('sticky end'), especially preferably an overhanging 5' end which comprises 4 nucleotides and has no self-homology (palindromic sequence). As a result, a stable, monomeric secondary structure ('hairpin loop') may form. The overhanging 5' end serves for linking defined nucleic acids, antisense oligonucleotides, but preferably transcribable and replicatable genes.
2. In the apex of the 'loop', the oligonucleotide carries a modified base which carries a grouping reactive with respect to the cross-linker, preferably an amino-modified 2'-deoxythymidine. In this case, the amino function of this modified base enables the linkage reaction between MBS and oligonucleotide.

The chimerical peptide-nucleic acid fragment is suitable for appropriately introducing nucleic acids into cells and cell organelles (e.g. nucleus, chloroplast), particularly for introducing ribonucleic acids (mRNA, 'antisense' oligonucleotides) and deoxyribonucleic acids (complete gene, 'antisense' oligonucleotides). It is especially suitable for the introduction of transcribable and processable genes into mitochondria, but even more suitable for the introduction of replicative, transcription-active and processable linear-cyclic nucleic acids (plasmids).

In a preferred embodiment, a transcribable gene is linked to the nucleic acid, containing the reactive linkage site or to the chimerical peptide-nucleic acid fragment. This is effected preferably the amplification of a gene, preferably a cloned gene consisting of a mitochondrial promoter, preferably the promoter of the light DNA strand (O_L, nt 490 -nt 369) and the gene to be expressed in a processable form, preferably a mitochondrial gene, preferably a mitochondrial transfer RNA, preferably the mitochondrial $tRNA^{Leu(UUR)}$ (nt 3204 - nt 3345) (S. Anderson *et al.* (1981), "Sequence and organization of the human mitochondrial genome", *Nature* 290:457-465). Following the enzymatic amplification of the gene, the linkage to the nucleic acid, containing the reactive linkage site, or to the chimerical peptide-nucleic acid fragment can be effected via a 'blunt end' ligation, but preferably a 'sticky end' ligation. For this purpose, the nucleic acid to be linked has at least one end capable of linkage, which consists preferably of a 5' overhand which comprises 4 nucleotides and has no self-homology (palindromic sequence). If both ends are to be linked with 'hairpin loops', a nucleic acid will preferably be selected which had differing 5' overhangs which comprise 4 nucleotides and have no self-homology. It is especially preferred to use nucleic acids whose 5' ends also have no homology with respect to one another. For the modification of the ends (cyclization) it is then preferred to use two different 'hairpin loops', one being specific (complementary) to the 'left" plasmid end and the other being specific to the 'right' plasmid end of the nucleic acid. In order to increase the stability of the nucleic acid over cellular and extracellular nucleases, the phosphorus diester bonds of the nucleic acid can be substituted with phosphorus thioate bonds and thus be protected if modified phosphorus thioate nucleotides have been used already in the enzymatic amplification.

A process comprising the following steps is suitable for the production of a chimerical peptide-nucleic acid fragment:

(a) Reaction of a nucleic acid (oligonucleotide), containing a functional linkage group, with a linkage agent.

(b) Reaction of the construct resulting from (a) with amino acids at the carboxy-terminal end of a peptide, containing a signal sequence, with the exception of a KDEL signal sequence, and

(c) optional extension of the chimerical peptide-nucleic acid fragment resulting from (b) by further DNA or RNA fragments.

In another preferred embodiment, the chimerical peptide-nucleic acid fragment can be produced by the following steps:

(a) Optional extension of the nucleic acid, containing a functional linkage group, by further DNA or RNA fragments.

(b) Reaction of the nucleic acid with functional linkage group or the extended nucleic acid resulting from (a) with a linkage agent.

(c) Reaction of the construct resulting from (b) with amino acids at the carboxy-terminal end of a peptide, containing a signal sequence, with the exception of a KDEL sequence.

In another embodiment which is a linear-cyclic nucleic acid in the form a plasmid, the selection of the nucleic acid depends on the genetic information which shall be expressed in which cell and in which aimed compartment of the cell. In this connection, nucleic acids which are to be transcribed have to have a suitable promoter. For example, if a gene is to be expressed in the mitochondrial matrix, a mitochondrial promotor can be chosen, preferably the promotor of the light mtDNA strand. The transcription is controlled in other cell compartments (e.g nucleus, chloroplast) by compartment-specific promoters.

The transcription is usually regulated by what is called transcription regulation sequences, preferably mitochondrial transcription regulation sequences. In general, these sequences comprise at least binding sites for factors which initiate the transcription (transcription initiation factor) as well as the binding site for the RNA synthesis apparatus. If a transcription is to be initiated in the mitochondria, binding sequences of

the mitochondrial transcription factors and of the RNA polymerase, particularly of the mitochondrial transcription factor 1 and the mitochondrial RNA polymerase, will be suitable. In other cell compartments (e.g. nucleus, chloroplast), the transcription can be controlled by compartment-specific transcription-regulation sequences.

In order to be able to regulate the transcription, the plasmid has transcription regulation sequences which are attached preferably in the 3' direction of the transcription initiation site (promoter). For example, if the transcription of a mitochondrial transformation plasmid is to be regulated, the control elements will be suitable for the H-strand and L-strand transcription of the mitochondrial genome, however preferable would be the so-called 'conserved sequence blocks' which terminate the transcription of the L-strand and simultaneously enable the transition of the DNA replication. In order to induce the exclusive transcription of the desired gene (optionally the desired genes in a polycistronic transcription), the transcription is discontinued on a suitable site behind the 3' end of the expressive gene/genes. This is achieved by the insertion of a suitable transcription-termination site, preferable arranged in the 3' direction to the promoter. For the regulated expression, the binding sequence for a bidirectionally acting transcription-termination factor is especially suitable in this case. For the transcription-termination in the mitochondria, a binding motif of a mitochondrial transcription-termination factor is preferably chosen here. At the same time, the formation of 'antisense RNA' of the head-to-head-linked dimeric plasmids is suppressed by the use of the transcription-termination factor binding sequence.

The selection of transformed cells can be controlled via the expression of a reporter gene. Expressive genes whose expression result in a macroscopic change of the phenotype are especially suitable as reporter or selection gene. A selection is made among genes which produce resistances to antibiotics, for example. In particular, the resistance genes for oligomycin (OLI) or chloramphenicol (Cap) are suitable for the use in a mitochondrial transformation system. In this connection, the mitochondrial chloramphenicol resistance gene appears to be a particularly suitable selection gene, since CAP-sensitive cell lines already change their phenotype at a portion of about 10% of the 16 S $rRNA^{CAP+}$ gene.

The replication of the nucleic acid can be realized by an initiation site for the DNA replication (replication origin). Therefore, the chimerical peptide-nucleic acid fragment in the form of a plasmid has to have at least one replication origin. In this connection, the orientation of the replication origin can be arranged irrespective of the expressive gene (genes), but preferably the replication origin is arranged in the 3' direction of the promoter. A suitable replication origin for a mitochondrial transformation plasmid would be a mitochondrial replication origin. In particular, the origin of replication of the heavy mtDNA strand is suitable in this case. It preferably has at least one 'conserved sequence block'. The replication can be controlled via what is called regulation sequences for the replication. For this purpose, the plasmid has to have at least one such sequence motif which is preferably arranged in the 3' direction of the promoter and the replication origin. Of the replication in the mitochondria is to be regulated, a mitochondrial replication regulation sequence will be especially suitable. It is preferred to use a motif which comprises at least one of the 'termination associated sequences'. In other cell compartments (e.g. nucleus, chloroplast), the replication is initiated at least via one compartment-specific replication origin and controlled via compartment-specific replication origin and controlled via compartment-specific replication regulation sequences.

In order to permit cloning of different genes into the plasmid molecule, the plasmid nucleic acid also has to have a suitable cloning module (multiple cloning site) which has the most widely differing recognition sequences for restriction endonucleases. Here, rare recognition sequences which do not occur on other sites of the plasmid are especially suitable. The cloning module can be incorporated into any site of the transformation plasmid. If the region of the cloning site is to be integrated into the transcription of the selection gene, the insertion of the multiple cloning site in the 3' direction of the promoter and in the 5' direction of the transcription termination site will be suitable. The integration of the multiple cloning site in the 5' direction of the selection gene is especially suitable, since in this case the use of the selection system is simultaneously accompanied by transcription of the region of the multiple cloning site.

In order to permit the autonomous replication in every aimed compartment of a cell when a nucleic acid is used, it has to be ensured that, after the synthesis of the

daughter strand, the DNA replication enzymes return to the synthesis starting point again to guarantee the covalent linkage of the 3' end with the 5' end of the newly synthesized daughter stand by corresponding enzymes. For this purpose, a linear nucleic acid plasmid is suitable which can be converted into a cyclic nucleic acid. The plasmid ends can be cyclized via the use of what is called ligation-capable (phosphorylated) end of nucleic acid. For this purpose, the use of a 'blunt end' nucleic acid or a nucleic having a overhanging 3' ends, but preferably a nucleic acid having overhanging 5' ends is particularly suitable. In this case, the overhanging ends should comprise at least one nucleotide. However, it is preferred to use overhanging 5' ends which are formed of four nucleotides. They have preferably no self-homology (palindromic sequence) and are also preferably not complementary to one another in order to suppress the formation of dimers in a subsequent nucleic acid linkage.

The cyclization of the prepared plasmid ends is arranged by synthetic oligonucleotides. They have a partial self-homology (partially palindromic sequence) and are thus capable to form what is called 'hairpin loop' structures. The partially palindromic sequence results in the formation of a stable, preferably monomeric secondary structure ('hairpin loop') having a blunt 5'-3' end (blunt end), and overhanging 3' end ('sticky end'), but preferably an overhanging 5' end. These oligonucleotides are especially preferred when they have a phosphorylated 5' end. When synthetic oligonucleotides having 'hairpin loop' structure are used, the linear plasmid DNA can be converted into a linear-cyclic system. The ends of the two oligonucleotides are preferably complementary to one end of the prepared plasmid nucleic acid each. For this purpose, two different 'hairpin loops' are preferably used, one being specific (complementary) to the 'right' plasmid end to suppress the dimer formation. At least one of the two 'hairpin loop' oligonucleotides may have at least one modified nucleotide. It guarantees the linkage site to a signal peptide, so that the nucleic acid transport can be arranged via the protein import route. In the model case, this linkage site (modified nucleotide) is placed at one of the unpaired positions of the 'loop'. A chemically reactive group, particularly an amino or thiol function is especially suitable as linkage site.

In order to prepare the ends of the transformation plasmid of the modification (cyclization), it has to be ensured that the plasmid ends are complementary to the ends of the oligonucleotides ('hairpin loops'). On the one hand, this succeeds by amplifying the plasmid DNA with suitable oligonucleotides which ave at least one recognition sequence for a restriction endonuclease. In this case, recognition sequences for restriction endonucleases are suitable which do not occur repeatedly in the plasmid sequence. Especially suitable is the use of recognition sequences for restriction endonucleases generating overhanging ends ('sticky ends'), particularly those which produce overhanging 5' ends, preferably outside the own recognition sequence. In this connection, the recognition sequence for the restriction endonuclease *Bsa* I ($GGTCTCN_1N_5$) is especially suitable. On the other ahdn, the use of a cloned nucleic acid which already has the recognition sequences for a restriction endonuclease, preferably *Bsa* I, is suitable. As a result, the enzymatic amplification can be omitted and the nucleic acid obtained by plasmid preparation/restriction enzyme treatment can be used directly. It is preferred that the cloned nucleic acid already includes the recognition sequence for the restriction endonuclease *Bsa* I at both ends.

Various methods are available for purifying the transformation plasmid. Here, the main objection is to separate the cyclic plasmid molecule from the unreacted enducts. The use of DNA-degrading enzymes are proved to be suitable in this connection. In particular, it is recommended to use enzymes which ave a 5'-3' or 3'-5' exonuclease activity. Particularly the use of the exonuclease III leads to the complete hydrolysis of unreacted educts while the cyclic plasmid DNA remains intact (no free 5' ends or 3' ends). The reaction products can be purified either via electrophoretic or chromatographic processes but also by precipitation. A selection can be made among different purification processes. On the one hand, the cyclic nucleic acid conjugated with the linkage agent and the signal peptide can be treated with an exonuclease, preferably exonuclease III, and then be purified via chromatographic electrophoretic purification and precipitation, respectively. On the other hand, the cyclic plasmid DNA can also be treated with an exonuclease, preferably exonuclease III, be purified and subsequently be conjugated with the linkage agent and the signal peptide and be purified via a chromatographic, electrophoretic purification and precipitation, respectively.

The linkage with a signal peptide can be realized by means of modified oligonucleotides. This peptide directs *in vivo* the transformation plasmid into the desired cell compartment. To this end, either the transformation plasmid can first be reacted with the modified oligonucleotide (ligation) and then the conjugation with the linkage agent and the signal peptide can take place or the modified oligonucleotide is first conjugated with the linkage agent and the signal peptide an then be used for the cyclizing the transformation plasmid ends (ligation).

The transformation system (cellular transformation) can overcome the cell membrane by various methods. Here, 'particle gun' system or microinjection are suitable, but electroporation and lipotransfection are preferred. All methods ensure the introduction of the linear-cyclic peptide nucleic acid plasmid into the cytosol of the cell from where the plasmid is directed to its site of action (aimed compartment) by the conjugated signal peptide.

As compared to the prior art transformation and infection methods, mentioned in the introductory part of the description, this process offers, for the first time, the possibility of appropriately introducing nucleic acids into cells and cell organelles. The selection of the signal sequence can determine the aimed compartment which is to be reached in this case (cytosol, nucleus, mitochondrion, chloroplast, etc.). Along with the compartment-specific and cell-specific introduction behavior, this process distinguishes itself by its universal applicability. Both prokaryotic and eukaryotic cells and cell systems can be treated with the translocation vector. Since a natural transport system of the membranes is used for the appropriate introduction, the treatment of cells or cell organelles with membrane-permeabilizing agents becomes superfluous (e.g. calcium chloride method, see above).

When a replicative and transcription-active nucleic acid is used, the plasmid does not unfold its full size until the first replication cycle has been completed: As a genuine cyclic plasmid (artificial chromosome) it now has the double genetic information (head-to-head linked plasmid dimers). In particular with respect to the use of this system for a somatic gene therapy, this behavior is induced intentionally and of decisive importance, since the genes to be expressed have to compete with the defect genes of the cells. In addition to this highest possible effectiveness, the system distinguishes itself through the

fact that it does not have to be integrated into a genome via a recombination step, such as retroviral systems, so as to become replicative. As a result, uncontrollable side-effects (undesired recombination) are already suppressed to the highest possible degree from the start. Therefore the application of this plasmid system can be expected without great safety risk.

The below examples explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

VI. EXAMPLES

A. Example 1: Introduction of a Chimerical Peptide-Nucleic Acid Fragment Into the Mitochondria

The overcoming of the mitochondrial double membrane system with a DNA translocation vector was studied to prove that nucleic acids can be transported appropriately across membranes by the above-described process. For this purpose, the mitochondrial signal sequence of the ornithine transcarbamylase (A.L. Horwich *et al.* (1983), "Molecular cloning of the cDNA coding for rat ornithine transcarbamylase", *Proc. Natl. Aca. Sci. U.S.A.* 80:4258-4262) (enzyme of urea cycle, naturally localized in the matrix of the mitochondria) was chemically prepared and purified. The original sequence was extended by a cysteine at the C terminus as reactive group for the subsequent linkage with the DNA (see fig. 1). This ensured that the heterobifunctional cross-linker (MBS) can only be linked with the thiol group of the only cysteine. A DNA oligonucleotide (39 nucleotides) were chosen as linkage partner. It distinguishes itself by two special features:

1. The sequence is partially palindromic and has and overhanging, phosphorylated 5' end (see fig. 1). As a result, what is called a 'hairpin loop' can form. The overhanging 5' end serves for ligating to this oligonucleotide defined nucleic acids which can then be imported into the mitochonndria.

2. The oligonucleotide carries a modified base in the vertex of the 'loop' (see fig. 1). In this case, an amino-modified 2'-deoxythymidine is concerned (see fig. 2). Here, the amino function of the modified bases in this connection enables the linkage reaction between MBS and oligonucleotide.

The tree reaction partners (oligonucleotide, MBS and peptide) are linked in individual reaction steps. Firstly the oligonucleotide (50 pmoles) is reacted in a buffer (100 μl; 50 mM potassium phosphate, pH 7.6) with MBS (10 nmoles dissolved in DMSO) (reaction time: 60 min.; reaction temperature: 20°). Unreacted MBS is separated via a Nick-spin column^R, (Sephadex G 50, Pharmacia) which was equilibrated with 50 mM of potassium phosphate (pH 6.0). The eluate contains the desired reaction step with the peptide (2.5 nmoles) (reaction time: 60 min.; reaction temperature 20°C). The reaction was stopped by the addition of dithiothreitol (2 mM). The linkage product (chimera, see fig. 3) was separated via a preparative gel electrophoresis of unreacted educts and isolated from the gel by electroelution (see fig. 4). Differing nucleic acids can now be linked by simple ligation to the overhanging 5' end of the oligonucleotide.

A 283 bp long double-stranded DNA (dsDNA) was amplified via an enzymatic reaction (PCR) in the below experiment. For this purpose, a DNA fragment cloned in to pBluescript^R (Stratagene) served as template DNA, which fragment in addition to the human mitochondrial promoter of the light strand (P_L, nt 902 - nt 369) included the gene for the mitochondrial transfer RNA leucine ($tRNA^{Leuc(UUR)}$, nt 3204 - nt 4126) (see fig. 5). Two oligonucleotides served as amplification primers, primer 1 having a non-complementary 5' end (see fig. 5). The dsDNA was modified by the 3'-5' exonuclease activity of the T4 DNA polymerase (incubation in the presence of 1 mM dGTP) which can produce overhanging 5' ends under conditions with which a person skilled in the art

is familiar (C. Aslanidis *et al.* (1990), "Ligation-independent cloning of PCR products (LIC-PCR)", *Nucleic. Acids Res.* 18:6069-6074).

Together with the previously conjugated peptide-MBS oligonucleotide the PCR-amplified DNA could be joined using the T4 DNA ligase. In order to be able to easily detect the linkage partners after the introduction into the mitochondria, the free 5'-OH group of the ligated DNA was phosphorylated radioactively by an enzymatic reaction (A. Novogrodsky *et al.* (1966), "The enzymatic phosphorylation of ribonucleic acid and deoxyribonucleic acid, I. Phosphorylation at 5'-hydroxyl termini", *J. Biol. Chem.* 241:2923-2932; A. Novogrodsky *et al.* (1966), "The enzymatic phosphorylation of ribonucleic acid and deoxyribonucleic acid. I. Further properties of the 5'-hydroxy polynucleotide kinase", *J. Biol. Chem.* 241:2933-2943).

A fresh rate liver was comminuted for the isolation of mitochondria, suspended in 25 mM HEPES, 250 mM saccharose, 2 mM EDTA, 52 μM BSA and homogenized in a glass homogenizer (50 ml). Cell membranes, cellular debris and nuclei were centrifuged of fat 3000 g and the supernatant was prepared for another centrifugation. For this purpose, the supernatant was placed in cooled centrifuge cups and centrifuged at 8000 g. The isolation mitochondria were resuspended in 200 ml of the same buffer and centrifuged again at 8000 g. The purified mitochondria pellet was resuspended in an equal volume of the same buffer and energized by the addition of 25 mM succinate, 25 mM pyruvate and 15 mM malate. The protein content of the suspension was determined by a Bradford Testkit^R (Pierce). 200 μg of mitochondrial protein (energized mitochondria) were incubated together with 10 pmoles of the chimera at 37°C for 60 min. (0.6 M sorbitol 10 mM potassium phosphate pH 7.4, 1 mM ATP, 2 mM $MgCL_2$, 1 % BSA). The mitochondria were reisolated by centrifugation at 8000 g, resuspended in 0.6 M sorbitol, 10 mM potassium phosphate pH 7.4, 2 mM $MgCL_2$, 1 % BSA, 10 U/ml DNAse I and incubated at 37°C for 30 min. This washing step was repeated twice to remove non-specifically adhering molecules. For proving that the chimera is associated with the mitochondria, the re-isolated mitochondria were purified via sucrose gradient density centrifugation. The individual fractions of the gradient were analyzed to localize the chimera and the mitochondria. The adenylate kinase which determines cytochrome-c oxidate and malate dehydrogenase activity was used as marker for the

mitochondria, while the chimera could be identified via the ^{32}p radiation measurement (see fig. 6). An analog experiment for determining the non-specific DNA introduction was carried out with the same DNA which was not linked with the signal peptide (see fig. 6). It was derived from the measurements that 65% of the chimera used segregated specifically with the mitochondria, whereas the non-specific DNA incorporation was less than 5% of the DNA used. In order to show that the chimera is not only associated with the surface of the mitochondria (membrane, import receptor), the re-isolated mitochondria were not fractioned into the three compartments of outer mitochondria membrane/intermembranous space, inner mitochondrial membrane and matrix space. For this purpose, the mitochondria were incubated with digitonin (final concentration: 1.2% w/v digitonin) and the resulting mitoplasts were separated via a sucrose gradient density centrifugation, collected in fractions and the activities of marker enzymes (adenylate kinase: intermembranous space, cytochrome c oxidase: inner mitochondria membrane; malate dehydrogenase; matrix space) were determined according to Schnaitman and Grennawalt (C. Schnaitman *et al.* (1968), "Enzymatic properties of the inner and outer membranes of rat liver mitochondria", *J. Cell Biol.* 38:158-175; C. Schnaitman *et al.* (1967), "The submitochondrial localization of monoamine oxidase. An enzymatic marker for the outer membrane of rat liver mitochondria", *J. Cell Biol.* 32: 719-735) (see fig. 7). An analog experiment for determining the non-specific DNA incorporation was carried out with the same DNA which was not linked with the signal peptide (see fig. 7). It was derived from the measurements that 45% of the chimera are associated with the mitoplasts, whereas the non-specifically adhering DNA could be assessed to be less than 3%. The isolated mitoplasts (less of the outer membrane and the intermembranous space) were lyzed by Lubron^{R} (0.16 mg/mg protein; ICN) and separated into the compartments of inner mitochondrial membrane (pellet) and matrix space (supernatant) by ultracentrifugation at 144,000 g. The compartments were assigned via the measurement of the activities of the cytochrome c oxidase (inner mitochondrial membrane) and the malate dehydrogenase (matrix space). The chimera was measured via the detection of the ^{32}p radiation in the scintillation counter and the result was 75% segregation with the matrix of the mitochondria, while 25% of the

chimera remained associated with the inner membrane of the mitochondria (incomplete translocation).

B. Example 2: Incorporation of a Replicative and Transcription-Active Chimerical Peptide-Nucleic Acid Fragment (Plasmid) Into the Mitochondria of Living Cells

In order to prove that a linear peptide-nucleic acid plasmid having cyclic ends ('hairpin loops') can overcome membranes *in vivo* via the protein import route and can be transcribed and replicated in spite of the chemical linkage with a signal peptide, the transcription and replication behavior were studies after the transfection of cells and the import into the matrix of the mitochondria. For this purpose, the signal peptide of the mitochondrial ornithine transcarbamylase was prepared synthetically, purified and linked with a nucleic acid plasmid.

Precondition for the examination of the correct transcription and replication behavior is the physical structure of the plasmid: for the experiment described below, a 3232 bp long double-stranded vector DNA (dsDNA) was cloned into pBluescript^R (Stratagene). For this purpose, the region of the mitochondrial genome was amplified via two modified oligonucleotides (primer 1, hybridized with the nucleotides 15903-15924 of the human mtDNA, includes at the 5' end an extension by the sequence TGTAGctgcag for the incorporation of a *PstI* site; primer 2, hybridized with the nucleotides 677-657 of the human mtDNA, includes at the 5' end an extension by the sequence TTGCATGctcgagGGTCTCAGGG for the incorporation of the *XhoI* site), which comprised the promoter of the light DNA strand, the regulation motifs for the transcription (CSBs, 'conserved sequence blocks') as well as the regulation site for the DNA replication ('TAS', termination associated sequences, (D.C. Wallace (1989), "Report of the committee on human mitochondrial DNA", Cytogenet. *Cell Genet.* 51:612-621) (see fig. 8). A multiple cloning site (MCS/TTS) was produced via a chemical synthesis of two complementary oligonucleotides (MCS/TTS 1 and 2) which contain the recognition sequences for various restriction endonucleases (see fig. 9). Under conditions with which a person skilled the art is familiar, the two oligonucleotides form hybrids which, after the phosphorylation with T4 DNA polynucleotide kinase, can be used for the ligation. In this connection, the hybrids distinguish themselves by 5' and 3' single-stranded-overhanging ends which are

complementary to a *Pst* I, on the one hand, and are complementary to a *Bam* HI site, on the other hand (see fig. 9). Together with the multiple cloning site, the synthetic oligonucleotides MCS/TTS 1 and 2 also comprise a bidirectional mitochondrial transcription termination sequence (see fig. 9). It is arranged in the 3' direction of the MCS and ensures that the transcription on this site is discontinued thus correctly forming terminated transcripts forming. This sequence motif also ensures that in the cyclic plasmid system no 'antisense RNA' is expressed. The ligation reaction between pBluescript, PCR-amplified fragment and the MCS/TTS hybrids took place in a stoichiometry of 1;2:2 under conditions with which a person skilled in the art is familiar. After the transformation, several E. coli colonies (clones) could be isolated and characterized. For this purpose, the corresponding plasmid DNA was subjected to dideoxy sequencing (fig. 10) under conditions with which a person skilled in the art is familiar.

For the experimental examination of the replication and transcription, what is called a reporter gene was inserted in the multiple cloning site. The chloramphenicol-resistant human mitochondrial 16 S ribosomal RNA was chosen as the reporter gene. It distinguishes itself from the naturally occurring ribosomal RNA only by a modified nucleotide (polymorphism). By means of the polymerase chain reaction, a fragment having two modified ologonucleotides (primer 3, hybridized with the nucleotides 1562-1581 of the mitochondrial DNA, extended at the 5' end of the sequence CCTCTaagctt for the incorporation of a *Hind* III site; primer 4, hybridized with the nucleotides 3359-3340, extended at the 5' end of the sequence GCATTactagt for the incorporation of a *Bcl* I site) was amplified from a DNA extract of chloramphenicol-resistant HeLa cells under conditions with which a person skilled in the art is familiar. In order to ensure a correct processing of the subsequent transcript, the amplification product included the two flanking tRNA genes ($tRNA^{Val}$ and $tRNA^{Leu}$). The amplified DNA was treated with the restriction endonucleases *Hind* II and *Bcl* I, purified by precipitation and used with the pBluescript plasmid 1 treated with *Hind* III and *Bcl* I (see figs. 8, 9 and 10) in a stoichiometry of 1:1 in a ligation reaction under conditions with which a person skilled in the art is familiar. The cloning strategy is illustrated in fig. 11.

Several E. coli colonies (clones) could be isolated and characterized. For this purpose, the corresponding plasmid DNA was subjected to a dideoxy sequencing under conditions with which a person skilled in the art is familiar (see fig. 12). In order to prepare the cloned DNA for the application to cell cultures and mitochondria, the cloning insert (mitochondrial transformation plasmid) was separated by the use of the restriction endonuclease *Bsa* I from the pBluescript vector under conditions with which a person skilled in the art is familiar. Alternatively, the insert DNA could be amplified via two oligonucleotides (primers 2 and 5; nucleotide sequence of primer 5: GATCCGGTCTCATTTTATGCG) by the polymerase chain reaction. The use of S-dNTPs permitted the production of 'thionated' DNA which is stabilized over cellular nucleases. In both cases, the subsequent use of the restriction endonuclease *Bsa* I resulted in two different 5' overhangs. They are complementary to the 'hairpin loops' used in order to achieve a cyclization of the linear nucleic acid (see figs. 13a and b). The oligonucleotides are produced via chemical synthesis. As a result, they do not have phosphorylated 5' ends and have to be phosphorylated by a kinase reaction under conditions with which a person skilled in the art is familiar (in order to be able to subsequently examine the cellular transformation, [γ-^{32}P]-ATP was partially used in this reaction as a substrate to radioactively label the plasmid). A majority of the 'hairpin loop' structure of the oligonucleotides forms spontaneously, since the palindromic sequence can hybridize with itself. However, dimers of the 'hairpin loops' can also be converted into monomers by denaturing them in the greatest possible volume (<0.1 μM) at 93°C for at least 5 min. and fixing them immediately in a solid matrix by freezing. Then, the oligonucleotides are slowly thawed at 4°C and then 99% thereof are available in the desired monomeric 'hairpin loop' structure (see fig. 14).

The plasmid DNA was cyclized together with the two monomerized 'hairpin loops' (HP 1 and 2) in a reaction batch. In this case, the molar ratio plasmid DNA to the two 'hairpin loops' was 1:100:100 (plasmid:HP1:HP2). By using the T4 DNA ligase, the individual reactants could be combined under conditions with which a person skilled the art is familiar (see fig. 15). The ligation products were purified by a treatment with exonuclease III (reaction conditions: 37°C, 50 min.). While nucleic acids having free 3' ends are decomposed by the nuclease, the plasmid DNA linked with

the two 'hairpin loops' remains stable over the 3'-5' exonuclease activity of the enzyme. The only reaction product (see fig. 15a) was separated via a preparative agarose gel electrophoresis and purified by an electroelution or by using QIAquick (Qiagen) in accordance with the manufacturer's recommendation.

The ligation product was examined via an RFLP analysis (restriction fragment length polymorphism). For this purpose, the ligated and purified plasmid DNA was treated with the restriction endonuclease *Mae* III under conditions with which a person skilled in the art is familiar. The DNA had five cleavage sites, so that fragments of differing dizes form which can be analyzed via an agarose gel (4%). Fig. 15b shows by way of example the *Mae* III cleavage pattern that is obtained after the ligation of the plasmid DNA with the two 'hairpin loops'. In this case, the DNA bands marked by the arrow tips represent the left and right end of the amplified (lane 1) and the linear-cyclic (lanes 2 and 3) mitochondrial plasmids.

For the conjugation of the circularized plasmid with teh synthetic signal peptide of the rate orninthine transcarbamylase (H_2N-MLSNLRILLNKAALRKAHTSMVRNFRYGKPVQSQVQ-LKPRDLC-COOH), the nucleic acid was incubated with 20 times a molar excess of m-maleimidobenzoyl-N-hydroxysuccinimide ester (linage agent) at 20°C for 60 min. (incubation medium: 50 mM potassium phosphate pH 7.8 The excess linkage agent was separated by a 'nick spin column' (Pharmacia-LKB) under conditions with which a person skilled in the art is familiar. The 'activated' nucleic acid was conjugated by reacting the nucleic acid with 50 times the molar excess of the signal peptide at 20°C (incubation medium: 50 mM potassium phosphate pH 6.8). The reaction was stopped by the addition of 1 mM dithiothreitol after 45 min. and the conjugate was available for the experiments to come.

In order to be able to show the *in vivo* usability of the peptide-nucleic acid plasmid, the plasmid had to be incorporated into eukaryotic cells. For this purpose, a chloramphenicol-sensitive B lymphocyte or fibroblast cell culture was transfected via a lipotransfection with the peptide-nucleic acid plasmid (the labeling was introduced at ^{32}p labeling during the kinase reaction of the 'hairpin loop' (HP1)) was pre-incubated together with 2-6 µl serum-free Optimem^R (Gibco-BRL) (20°C, 15 min.). During the incubation the polycationic lipid of the LipfectAmine^R reagent DOSPA (2,3-dioleyloxy-

N-[2-(sperminecarboxamido)-ethyl]-N,N-dimethyl-1-propaneaminiumtrifluoroacetate) forms unillamellar liposomes with the aid of the neutral lipid DOPE (dioleoylphosphatidylethanolamine), which can complex the DNA. Then, the reaction batch was added to the prepared cells, adjusted to a density of about $2.5*10^6$ cells per 0.8 ml (35 mm culture dishes, 4 h, 37°C, CO_2 incubator). The transfection medium was then replaced by 5 ml of DMEM medium (Gibco-BRL) previously supplemented by 10% fetal calf serum and 100 µg/ml chloramphenicol. The transformation efficiency was determined by the measurement of the ^{32}P radiation of the construct. As a rule, a cellular incorporation rate of 80-85% of the chimerical construct were associated with the transformed cells and 15-20% of the chimerical peptide-DNA plasmid remained in the supernatant of the transfection reaction.

After about 21-28 days, chloramphenicol-resistant colonies formed in the transformed cells. Under conditions with which a person skilled in the art is familiar, the resistant cells were isolated and multiplied. Under conditions with which a person skilled in the art is familiar, sufficient DNA could be obtained from about $1*10^5$ cells to classify the genotypes. For this purpose, the isolated DNA was separated via agarose gel electrophoresis and transmitted to a nylon membrane (Southern blot). The nucleic acids were detected by hybridization using a specific, radioactively labeled probe (see fig. 16). In addition to the introduced circularized 'linear' vector (lanes 2 and 6) an 'in vitro' transcription (lane 3), an 'in vitro' replication (lane 4), as well as the intermediates obtained 'in vivo' (isolated nucleic acids of a transformed clone) are shown in this illustration. While the three smaller bands can be produced in vitro by incubating the circularized vector with the four nucleoside triphosphates (RNA) and a mitochondrial enzyme extract (lane 3), the formation of a dimer, circular plasmid (greatest band in lane 4) is observed in the further addition of the deoxynucleoside triphosphates to the reaction batch; an identical image yields the analysis of the nucleic acids which can be obtained from transformed cell colonies (lane 5). The fact that the greatest DNA band in lanes 4 and 5 is actually the dimeric and thus replicated mitochondrial plasmid, could be confirmed by sequence analysis.

A lipotransfection batch were the non-conjugated plasmid not linked with the signal peptide was sued, served as a control experiment. As expected, this plasmid was

not incorporated into the mitochondria of the transfected cells and thus did not result in teh formation of chloramphenicol-resistant cells. These cells stopped growth after 10 days and decayed within the following 8 to 10 days completely.

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.